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㉗ Oxidation resistant IL-2 muteins and their production, formulations containing such muteins, and DNA sequences and expression vectors coding for such muteins and corresponding transformed host cells.

㉘ A biologically active reference (e.g. human) IL-2 (interleukin-2) protein is protected against oxidation by a method involving substituting a conservative amino acid for each methionine residue susceptible to chloramine T or peroxide oxidation and wherein additional, non-susceptible methionine residues are not so substituted.

**EP 0 200 280 A2**

OXIDATION RESISTANT IL-2 MUTEINS AND THEIR PRODUCTION, FORMULATIONS CONTAINING SUCH MUTEINS, AND DNA SEQUENCES AND EXPRESSION VECTORS CODING FOR SUCH MUTEINS AND CORRESPONDING TRANSFORMED HOST CELLS

The invention relates to the use of recombinant techniques to provide modified protein sequences resistant to oxidation, i.e., oxidation-receptive proteins. More specifically, the invention relates to providing proteins which retain full biological activity, but which contain conservative amino acid substitutions for specific methionine residues that are particularly susceptible to oxidation.

The fact that different proteins undergo varying degrees of modification during purification is well known, but not well understood. Certain proteins are known to be unusually susceptible to thermal denaturation or to proteolytic cleavage; others contain reactive amino acid side chains located in positions which render them particularly susceptible to chemical modification, including oxidation. In general, it is not possible to predict from the amino acid sequence the extent to which any of the above modifications will occur.

This problem is compounded because ordinary preparative methods for protein purification may lack the resolution to detect or separate small amounts of impurities. Such commonly employed techniques and criteria for homogeneity as electrophoresis, gel filtration, and ion exchange chromatography may fail to reveal the presence of small amounts of contaminating material which can be shown to be present by more sensitive analytical techniques, such as reverse phase high performance liquid chromatography (RP-HPLC).

Floor, E., et al., Anal. Biochem. (1980) 101:498-503 describes an illustration of this problem. An undecaprotein which has neurotransmitter properties, substance P, containing a carboxy terminal methionine that is oxidizable to methionine sulfoxide in dilute solutions exposed to air, elutes from ion exchange columns as a single peak, but reveals the presence of the sulfoxide-containing contaminant when subjected to RP-HPLC. Similarly, Frelinger, A.L., et al., J. Biol. Chem. (1984) 259:5507-5513 showed that bovine parathyroid hormone which appeared homogeneous by conventional criteria could be shown by RP-HPLC analysis to contain sequences with either one or both of the methionine residues oxidized to the sulfoxide. The proteins were eluted from RP-HPLC in an order directly proportional to the number of methionine residues oxidized.

Recently, Rosenberg, S., et al., Nature (1984) 312:77-80 produced a mutagenized human alpha-1 anti-trypsin in yeast which contained a valine residue in place of the methionine at the active site,

and which retained significant biological activity. This substitution was made to prevent the observed decrease in inhibitory activity toward elastase, caused by oxidation of this methionine, which destroys the protective function of alpha-1 anti-trypsin in the lungs. The valine substitution resulted in an active anti-trypsin which was, unlike the native form, resistant to oxidation by chemical oxidants which may be similar to those released by leukocytes or present in cigarette smoke. Hence, by making this substitution, it is believed that the levels of anti-trypsin needed to be administered could be reduced. Carrell, R., Nature (1984) 312:77-80 discloses uses of  $\alpha$ -antitrypsin mutants such as the Rosenberg et al. mutant in which methionine is replaced by valine. Transgene S.A. has replaced the methionine of  $\alpha$ -antitrypsin with arginine, presented on May 20, 1985.

When a methionine is located at or near an active site, as in alpha-1 anti-trypsin, and is clearly crucial to biological activity, its oxidation to the sulfoxide might be expected to be detrimental. However, the effects of residual oxidation during purification of proteins containing several methionine residues which are not at the active site is less clear. Data for a number of enzymes containing oxidized methionine residues show this oxidation to have varying degrees of negative effects on activity. At a minimum, the putatively pure protein obtained will, if the oxidized form is not separated away, contain a contaminant, and will therefore not be homogeneous. The effect of the presence of such contaminant(s) per se in, for example, pharmaceutical protein preparations, is undesirable. Further, the presence of an oxidized methionine may cause other structural changes in the desired protein, such as decreased solubility, increased proteolytic lability, or the inhibition of formation of a required disulfide bond, which may, in turn, have additional side effects, such as the formation of dimeric and higher aggregated forms. Taken together, this collection of impurities may be at least partly responsible for the often observed immunogenicity of parenterally administered proteins, or for other deleterious physiological reactions.

EP 0,130,756 published January 9, 1985 discloses replacing methionine at position 222 of subtilisin with an amino acid such as alanine to provide resistance to oxidation. Estell, D. et al., J. of Biol. Chem. (1985) 260:6518-6521 discloses mutant subtilisin enzymes whose methionine residues are susceptible to oxidation.

The present invention provides a method for protecting a human therapeutic protein which normally contains methionines from oxidation to the methionine sulfoxide during purification by substituting a conservative amino acid, e.g. alanine, for any methionine particularly susceptible to oxidation. Thus, protected, the mutein retains its biological activity, but has enhanced stability. It is not subject to contamination by either modified forms containing the methionine sulfoxide per se, or by side products whose formation is the result thereof.

The invention provides a means to protect specific methionine residues whose preferential oxidation results in therapeutic human protein sequences containing methionine sulfoxide residues. Even in small amounts, these impurities may be potential immunogens, and they detract from the homogeneity of the therapeutic product. Very small amounts of contaminants are significant, especially in the context of drugs injected at high dosage, such as interferons and lymphokines. By providing a conservative amino acid substitution for the susceptible methionine, applicants believe that a majority of the impurities may be prevented.

Thus, in one aspect, the invention relates to a method for protecting a biologically active reference human therapeutic protein having IL-2 activity against oxidation comprising making a conservative amino acid substitution for each methionine residue susceptible to chloramine T or peroxide oxidation, other methionine residues which are significantly less susceptible not being so substituted. The resulting oxidation-resistant mutein has substantially the same activity as the reference protein, but is capable of resisting low level oxidation commonly encountered in purification or storage so as to provide a homogeneous preparation by the criterion of RP-HPLC and other analytical techniques which detect methionine sulfoxide. In other aspects, the invention relates to the muteins so produced, recombinant DNA sequences encoding the muteins, recombinant expression vectors comprising the DNA sequence operably linked to control sequences compatible with a suitable host, host cells transformed with the vector, and therapeutic formulations containing certain of the muteins so produced.

It is preferred to determine which of the methionine residues is particularly susceptible to oxidation before carrying out the conservative amino acid substitution. Of the four methionines in interleukin-2, the methionine at position 104 has been shown to be preferentially oxidized during conventional purification or exposure to chemical oxidants. Accordingly, in other aspects, the invention relates to IL-2 muteins which harbor conser-

vative amino acid substitutions in position 104 and to therapeutic formulations containing such muteins.

Figure 1 shows the amino acid sequence of native IL-2, and the position number designations used herein.

Figure 2 shows RP-HPLC analysis of both modified and reference IL-2 produced in *E. coli*, before and after chloramine T treatment.

Figure 3 shows RP-HPLC analysis of both modified and reference IL-2 produced in *E. coli*, before and after hydrogen peroxide treatment.

Figure 4 shows a Coomassie stained SDS-PAGE analysis of extracts or purified IL-2, both from transformed *E. coli* which produce either the IL-2 mutein of the invention or the reference IL-2.

Figure 5 shows the restriction map of pJDB219 which may be used as a yeast expression vector. This map represents the B form of this plasmid. In circle zero cells like *S. cerevisiae* C468 it will remain as the B form, since it contains no intact flip gene.

#### A. Definitions

As used herein, "reference" protein refers to a protein of known amino acid sequence whose biological activity is also known, and which contains at least one methionine which becomes oxidized upon treatment with chloramine T. It has been shown that chloramine T preferentially oxidizes, in native - (non-denatured) proteins, only methionine residues which are exposed (Shechter, Y., et al., *Biochem.* - (1975) 14:4497-4503). Treatment of peptides or of a denatured native protein, in at least some cases, results in oxidation of all methionine residues to the sulfoxides. Oxidation of any available sulfhydryl groups to disulfide linkages may also occur. Treatment with hydrogen peroxide under appropriate conditions also results in conversion of methionine to methionine sulfoxide.

"Oxidation-resistant mutein", in relation to the "reference" protein, refers to a related amino acid sequence which has a biological activity substantially the same as the reference protein. However, it contains a conservative amino acid substitution for any methionine residue which is particularly susceptible to oxidation by chloramine T or hydrogen

peroxide. Such substitutions are not intended to render the mutein resistant to all oxidations, such as beneficial oxidations which might include creation of a disulfide linkage required for full activity.

A methionine "susceptible to oxidation by chloramine T or peroxide" refers to a methionine which undergoes relatively rapid, i.e., detectable, conversion to the sulfoxide in the presence of either of these reagents under the conditions specified below. For treatment with either reagent the protein is dissolved in buffer, 50 mM Tris-HCl, pH 8.0, for chloramine T, 50 mM sodium phosphate, pH 6.8 for peroxide, at a concentration of about 0.2 mg/ml. For some recombinantly produced proteins, solubilizing agents, e.g., 0.1% detergent, may be required to effect solubility. For chloramine T, the oxidant is added to a two-fold molar excess, and the reaction incubated for 15 minutes at 25°C. For peroxide, H<sub>2</sub>O<sub>2</sub> is added to 30 mM and the reaction incubated for one hour at 25°C. Chloramine T oxidation and hydrogen peroxide oxidation exhibit overlapping specificities with respect to methionine. Significant (detectable) oxidation with respect to either of these reagents under the above specified conditions defines a methionine residue as "susceptible" for purposes herein.

A "non-susceptible" methionine residue is defined as a methionine residue which is oxidizable, but is not oxidized detectably (by the means used to detect susceptible methionines) under the conditions specified above, i.e., it is oxidized at a slower rate than the "susceptible" methionine residues. The methionines in proteins have variable susceptibility to oxidation, and the preferred compounds have the most susceptible methionine(s) replaced. The degree of susceptibility and the number of methionines to be replaced will depend on the protein. If it is possible to make the protein homogeneous without further methionine substitutions, then only the most susceptible methionine(s) will be replaced because it is desirable to keep the protein sequence as close to the native protein sequence as possible.

A "conservative" amino acid alteration is defined as one which does not adversely affect biological activity. Conservative amino acid substitutions for oxidizable methionines, according to the invention, are selected from the neutral or non-polar amino acids. Preferred are glycine, alanine, serine, threonine, valine, isoleucine, leucine, asparagine, glutamine, glutamate, tyrosine, and phenylalanine. More preferred are alanine, serine, threonine, valine, leucine, and isoleucine. Even

more preferred are alanine, serine, leucine, glutamate, and valine, and most preferred is alanine. Also included among conservative alterations is deletion of the methionine.

It should be noted that mouse IL-2, which shares 65% amino acid homology with human IL-2 overall and including the region near residue 104, has a glutamic acid substitution for methionine 104. Fiers, W. et al., in Cellular and Molecular Biology of Lymphokines, C. Sorg and A. Schimpl, editors, pp. 595-603, Academic Press, 1985. There is no near-by methionine in the mouse sequence, suggesting that methionine is not required at or near residue 104 for activity and that glutamic acid may also be an acceptable substitution at residue 104 in the human sequence.

The relationship, therefore, of the "reference" protein to the oxidation-resistant mutein is that of identical amino acid sequence except for substitution of a conservative amino acid for the susceptible methionine, or the deletion thereof.

"Recombinant host cells", "host cells", "cells", "cell cultures", and so forth are used interchangeably, and designate individual cells, cell lines, cell cultures and harvested cells which have been, or are intended to be, transformed with the recombinant vectors of the invention. These terms also include the progeny of the cells originally receiving the vector. It is well understood that not all of the progeny of a single cell are precisely, necessarily identical with the parent, due to spontaneous or intentional mutations or alterations in the culture conditions. These progeny are also included in the definition, so long as the capacity to perform the function of producing the oxidation-resistant mutein of the invention conferred by the vector is retained.

"Transformed" refers to any process for altering the DNA content of the host, including *in vitro* transformation procedures as described below, phage infection, or such other means for effecting controlled DNA uptake as are known in the art.

"Operably linked" as used herein regarding DNA sequences or genes refers to the situation wherein the sequences or genes are juxtaposed in such a manner as to permit their ordinary functionality. For example, a promoter operably linked to a coding sequence refers to those linkages where the promoter is capable of controlling the expression of the sequence.

"Control sequences" refers to DNA sequences which control the expression of the sequence which encodes the oxidation-resistant mutein. Examples include promoters for transcription initiation, optionally with an operator, enhancer regions, ribosome binding site sequences, and translation

signals which initiate and terminate translation of the gene. Such control sequences must be compatible with, i.e., operable in, the host into which they will be inserted.

"Inert, non-allergenic, pharmaceutically compatible carrier" refers to a carrier for the mutein herein which does not react with the mutein, is water soluble and preferably non-sensitive to water, is itself stable, does not elicit an allergic response in a patient, and is compatible with the mutein physiologically and pharmaceutically so that a stable, soluble formulation is made. The carrier may be liquid or solid and if solid, may be a solid bulking agent for pharmaceutical tablet formulation. The invention embraces both pharmaceutical and veterinary therapeutic formulations containing the present new muteins. Such formulations may be made in accordance with standard practice and may be in unit dosage form.

#### B. General Description

To obtain the oxidation resistant muteins of the invention, a reference protein is identified which exhibits preferential oxidation of a specific methionine residue and this residue is replaced by a conservative amino acid substitution. The invention is applied to proteins wherein this residue is known. Where the location of this residue is not known, it can be determined by means known in the art.

For example, RP-HPLC in some instances will be adequate to resolve reference protein from reference protein which contains methionine sulfoxide. The protein is subjected to analysis on RP-HPLC before and after treating with, e.g., chloramine T under the specific conditions set forth in 1A above. (Alternatively, peroxide could be used.) Conditions are selected whereby only the most susceptible methionine residues are oxidized to the sulfoxide. Both the major peak resulting from chloramine T oxidation, and the major peak obtained without such oxidation, are subjected to procedures designed to determine the position of any methionine residue which has undergone oxidation.

A variety of procedures is available. However, a particularly convenient procedure utilizes cyanogen bromide cleavage (which reagent cleaves at methionine residues, but is unreactive when those methionine residues are oxidized), followed by HPLC peptide mapping and sequence analysis.

The cyanogen bromide cleavage mixture is first reduced with, for example, dithioerythritol to destroy any disulfide linkages remaining. For the reference protein, untreated with chloramine T, the number of peptides obtained should equal the

number of internal methionine residues plus 1. For the protein oxidized by chloramine T, the number of resulting peptides will be diminished in proportion to the number of internal methionines oxidized. Thus, a comparison of the number of peptides obtained upon treatment by cyanogen bromide cleavage, DTE reduction, and HPLC analysis will permit a conclusion as to the number of internal methionine residues present, and the number which are exposed for oxidation by chloramine T. Sequencing of the peptides thus obtained, along with, for example, comparison to the complete amino acid sequence independently established, will then reveal the location of the oxidized methionine with respect to the complete sequence.

After the location of the susceptible methionine is established, the coding sequence for the protein is obtained and used for site-specific mutagenesis. The coding sequence for interleukin-2 is already known and available in the art.

To prepare an oxidation-resistant mutein of the reference protein, a DNA sequence encoding the reference protein, cloned into a convenient M-13 cloning vector, is subjected to site-specific mutagenesis using the appropriate primer to convert the residue at the identified position from methionine to a conservative amino acid replacement. Site-specific (or primer-directed) mutagenesis is now a technique which is well-established in the art. Briefly, a synthetic oligonucleotide complementary to the desired sequence is used as a primer in the synthesis of a strand complementary to the phage single-stranded reference sequence. The resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage. Theoretically, 50% of the plaques will consist of phage containing the mutated form; 50% will have the original sequence. The plaques are hybridized with kinased synthetic primer under stringency conditions which permit hybridization only with the desired sequence, which will form a perfect match with the probe. Hybridizing plaques are then picked and cultured, and the DNA is recovered.

The resulting DNA is then ligated into expression vectors using standard procedures, which can be precisely identical to those used in preparing expression vectors for the reference sequence. An oxidation-resistant mutein may then be produced in suitable hosts under the control of compatible control sequences using any of the recombinant host

cell systems known in the art (see ¶C.1). The methionine-replaced muteins thus produced are recovered and purified using standard protein purification techniques.

One protein purification technique if the protein is in the form of a refractile material produced from a microorganism host is to (a) disrupt the cell membrane of the host, (b) remove greater than 99% by weight of the salts from the disruptate, (c) redispersant the desalted disruptate, (d) add a material to the disruptate such as sucrose to increase the density or viscosity of, or to create a density or viscosity gradient in, the liquid within the disruptate, and (e) separate the refractile material from the cellular debris by high-speed centrifugation of 10,000 to 40,000 x g. Preferably the sucrose is added to increase the density of the liquid to a P of between 1.13 and 1.17 g/cm<sup>3</sup>.

Alternatively, the cell membrane may be disrupted and the disruptate is extracted with an aqueous solution of a chaotropic agent such as urea which extracts non-IL-2 proteins selectively from the cellular material and from the IL-2. Then the IL-2 is solubilized with a solubilizing agent such as SDS in the presence of a reducing agent to reduce the IL-2, the reduced IL-2 is then separated from the solution in the presence of the reducing agent, the IL-2 is then oxidized, purified by gel filtration or reverse-phase high performance liquid chromatography and recovered.

In a third embodiment, the first alternative is employed to isolate the refractile bodies and then the second alternative is employed to dissolve the refractile bodies in a solubilizing agent such as SDS in the presence of the reducing agent and separating, oxidizing and purifying as described above.

Oxidation of IL-2 during the recovery step may be carried out by using iodosobenzoic acid, as described by U.S. Patent No. 4,530,787, or by using a copper ion, such as copper chloride at a pH of about 5.5 and 9.

The oxidation-resistant preparations have similar biological activity to that of the pertinent reference protein, and therefore will be in the same applications.

#### B. IL-2 Oxidation-Resistant Muteins

The method of the invention is illustrated in one aspect by its application to interleukin-2, a lymphokine which is secreted from induced peripheral blood lymphocytes, or from Jurkat cells, as described by Taniguchi, T., et al., *Nature* (1983) 24:305.

Recombinant production of IL-2 has been reported by a number of groups. As shown in Figure 1, mature, native IL-2 has a 133 amino acid sequence containing alanine at position 1, three cysteines (at positions 58, 105, and 125), and four methionine residues (at positions 23, 38, 46, and 104). Designation of various forms of IL-2 herein is made with respect to the sequence shown and numbered as in Figure 1, noting only modifications thereof at the subscripted positions. Thus, the identical sequence, but missing the N-terminal alanine, is designated des-ala, IL-2; the same sequence but with a serine residue at position 125 (instead of cysteine as shown) is designated ser<sub>125</sub> IL-2.

In addition to native IL-2, recombinant forms of IL-2 which contain certain modifications in amino acid structure have been shown to be comparably active to the native sequence. For example, recombinant IL-2 lacking the five N-terminal amino acids, including the glycosylation site at position 3, has significant biological activity (S. Gillis, Immunex, December, 1984, personal communication). U.S. Patent No. 4,518,584 issued May 21, 1985, discloses a preferred modification of the N-terminal sequence with the alanine residue at position 1 deleted. Also disclosed are IL-2 muteins with a neutral amino acid residue such as serine, alanine, threonine or valine at position 125 instead of cysteine. These muteins have superior predictability with respect to disulfide linkage formation. Alternative conservative amino acid replacements at position 125, notably alanine or valine, are also disclosed. The applicants have found that both the ser<sub>125</sub> IL-2 and ala<sub>125</sub> IL-2 are fully active in cell proliferation assays. (Replacement of the cysteine residues at position 58 or 105 results in a loss of activity, since these residues are participants in the necessary disulfide link of the native molecule.)

Recombinant forms of IL-2 prepared for clinical testing have been shown to contain minor amounts of contaminants using analytical techniques with higher resolution than the preparative purification procedures used to manufacture the clinical samples. Both for proteins in general, and for IL-2 in particular, one cause for production of contaminants during purification, due to alteration of three-dimensional structure or formation of complexes, resides in the ability of certain free sulfhydryl groups to form disulfides other than those present in the active form of the protein. Accordingly, purification procedures for IL-2 are conducted under conditions which maintain cysteine residues in the sulfhydryl condition prior to permitting disulfide bond formation under controlled conditions of moderate oxidation. Nevertheless, it has now been found that because equipment and reagents used

in purification procedures for pharmaceutical proteins must be sterile, residual oxidants, such as hydrogen peroxide, from sterilizing wash solutions may be present in sufficient amounts to cause oxidative conversions in the IL-2 preparations. Furthermore, such oxidations may also occur on storage.

All of the above-mentioned forms of IL-2 may be used as the reference protein in this preferred embodiment which is designed to delete or substitute a conservative amino acid for the methionine residue susceptible to such oxidation. Oxidation-resistant muteins may be valuable in providing compositions which retain potency as protein products, or oxidation resistance may alter the pharmacological behavior of the molecules in other beneficial ways.

Activity for the oxidation-resistant IL-2 mutein is verified using the standard HT-2 cell bioassay, as set forth by Watson, J., J. Exp. Med. (1979) 150:1507-1519 and by Gillis, S., et al., J. Immunol. (1978) 120:2027-2032. The mutein is also equally effective at activating NK cells *in vitro* as the reference molecule.

The oxidation-resistant IL-2 mutein may be recovered from an RP-HPLC pool, obtained as described in 11B, by precipitating it from the pool - (which may consist of acidic propanol), neutralizing the pH as by adding base, thereby obtaining a precipitate, centrifuging, redissolving the centrifugate in a non-toxic solubilizer such as SDS, and using S-200 gel filtration to remove oligomers if necessary. When SDS is used as solubilizer, the SDS is reduced at the final formulation stage to a level of about 100 to 250, preferably approximately 200,  $\mu\text{g}/\text{mg}$  IL-2, by diafiltration using an appropriate buffer.

Following diafiltration, the IL-2 concentration is readjusted to a concentration in the range of about 0.01 to 2 mg/ml and the IL-2 may be formulated in a non-toxic, non-allergenic, pharmaceutically compatible carrier medium such as distilled water, Ringer's solution, Hank's solution, or physiological saline. A water-soluble carrier may be added to the desired level. The carrier will typically be added such that it is present in the solution at about 1 to 10% by weight, preferably about 5% by weight. The exact amount of carrier added is not critical. Conventional solid bulking agents which are used in pharmaceutical tablet formulation may be used as the carrier. These materials are water soluble, do not react with the IL-2, and are themselves stable. They are also preferably non-sensitive (i.e., nonhygroscopic) to water. Examples of carriers that may be added are non-toxic stabilizers such as mannitol or other materials such as lactose and

other reduced sugars such as sorbitol, starches and starch hydrolysates derived from wheat, corn, rice, and potato, microcrystallin celluloses, and albumin such as human serum albumin. Mannitol is preferred.

The carrier adds bulk to the formulation such that when unit dosage amounts of the solution are lyophilized in containers, such as sterile vials, the freeze-dried residue will be clearly discernible to the naked eye. In this regard the preferred carrier, mannitol, yields an aesthetically acceptable (white, crystalline) residue which is not sensitive to water. The nonsensitivity of mannitol to water may enhance the stability of the formulation.

After the carrier is added, the unit dosage amounts (i.e., volumes that will provide 0.01 to 2 mg, preferably 0.2 to 0.3 mg, IL-2 per dose) of the solution are dispensed into containers, the containers are capped with a slotted stopper, and the contents are lyophilized using conventional freeze-drying conditions and apparatus.

The lyophilized, sterile product consists of a mixture of (1) recombinant IL-2, (2) carrier - (mannitol), (3) detergent (SDS), and (4) a small amount of buffer which will provide a physiological pH when the mixture is reconstituted. The recombinant IL-2 will typically constitute about 0.015% to 3.85% by weight of the mixture, more preferably about 0.4% to 0.6% of the mixture. Storage tests of this product indicate that the IL-2 is stable in this form for more than three months at 2°C to 8°C.

The lyophilized mixture may be reconstituted by injecting a conventional parenteral aqueous injection such as water for injection, Ringer's injection, dextrose injection, dextrose and salt injection, or the like, into the vial. The injection should be added against the side of the vial to avoid excess foaming. The amount of injection added to the vial will typically be in the range of 1 to 5 ml, preferably 1 to 2 ml.

Administration of the IL-2 muteins to humans or animals may be, for example, intravenous, intraperitoneal, intramuscular, or subcutaneous as deemed appropriate by the physician. The amount of IL-2 mutein administered will usually range between about  $1 \times 10^4$  and  $2 \times 10^8$  units, depending on the subject and its weight.

The IL-2 muteins herein are useful for the diagnosis and treatment (local or systemic) of bacterial, viral, parasitic, protozoan and fungal infections; for augmenting cell-mediated cytotoxicity; for stimulating lymphokine-activated killer cell activity; for mediating recovery of immune function of lymphocytes; for augmenting alloantigen responsiveness; for facilitating recovery of immune function in acquired immune-deficient states; for reconstitution

of normal immunofunction in aged human and animals; in the development of diagnostic assays such as those employing enzyme amplification, radiolabeling, radioimaging, and other methods known in the art for monitoring IL-2 levels in the diseased state; for the promotion of T cell growth *in vitro* for therapeutic and diagnostic purposes; for blocking receptor sites for lymphokines; and in various other therapeutic, diagnostic and research applications.

Various therapeutic applications of human IL-2 have been investigated and reported by S. A. Rosenberg, and colleagues (see Mule, et al., *Science* (1984) 225:1487 and S. Rosenberg, et al., *New England Journal of Medicine* (1985) 313(23):1485-1492, for example). IL-2 muteins may be used by themselves or in combination with other immunologically relevant B or T cells or other therapeutic agents. Non-limiting examples of relevant cells are B or T cells, and natural killer cells; and exemplary therapeutic reagents which may be used in combination with the polypeptides of this invention are the various interferons, especially alpha-, beta-, and gamma-interferon, B cell growth factor, CSF-1, interleukin-1, or tumor necrosis factor.

### C. Standard Methods

Most of the techniques which are used to transform cells, construct vectors, effect hybridization with probe, and the like are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs may serve as a guideline.

#### C.1. Hosts and Control Sequences

Either procaryotic or eucaryotic hosts may be used for expression of DNA sequences; cloning of such sequences generally employs procaryotes for convenience. Procaryotes most frequently are represented by various strains of *E. coli*. However, other microbial strains may also be used, such as bacilli, for example *Bacillus subtilis*, various species of *Pseudomonas*, or other bacterial strains. In such procaryotic systems, plasmid vectors which contain replication sites and control sequences derived from a species compatible with the host are used. For example, *E. coli* is typically transformed using derivatives of pBR322, a plasmid from an *E. coli* species by Bolivar, et al., *Gene* (1977) 2:95. pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides additional

markers which can be either retained or destroyed in constructing the desired vector. Commonly used procaryotic control sequences include such commonly used promoters as the beta-lactamase - (penicillinase) and lactose (lac) promoter systems (Chang, et al., *Nature* (1977) 198:1056) and the tryptophan (trp) promoter system (Goeddel, et al., *Nucleic Acids Res.* (1980) 8:4057) and the lambda derived P<sub>L</sub> promoter and N-gene ribosome binding site (Shimatake, et al., *Nature* (1981) 292:128). Any available promoter system compatible with procaryotes can be used.

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of *Saccharomyces cerevisiae*, Baker's yeast, are most used, although a number of other strains are commonly available. Plasmid vectors suitable for yeast expression include use of the 2 micron origin of replication (Broach, J. R., *Meth. Enz.* (1983) 101:307), and those described by, for example, Stinchcomb, et al., *Nature* (1979) 282:39, Tschempe, et al., *Gene* (1980) 10:157 and Clarke, L., et al., *Meth. Enz.* (1983) 101:300). Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess, et al., *J. Adv. Enzyme Reg.* (1968) 7:149; Holland, et al., *Biochemistry* (1978) 17:4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.* (1980) 255:2073), as well as those for other glycolytic enzymes. Other promoters are available which have the additional advantage of transcription controlled by growth conditions such as the promoter regions for alcohol dehydrogenase 2, isocytocrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization (Holland, *ibid*). It is also believed that terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes such as those derived from the enolase gene containing plasmid peno46 (Holland, M. J., et al., *J. Biol. Chem.* - (1981) 256:1385) or the LEU2 gene obtained from YEp13 (Broach, J., et al., *Gene* (1978) 8:121).

The advantages of using yeast as a host for IL-2 production are that a homogeneous, native N-terminus (alanine) is achieved thereby, the protein is fully active, the protein need not be oxidized *in vitro* to form the 58 to 105 disulfide bond, because it is secreted in this form; the protein contains three cysteines as does the native protein, and the protein is more soluble than the recombinant IL-2 produced in *E. coli*. While the yeast-produced IL-2 polypeptide is more like IL-2 from native sources, it



is not produced in as great a yield as currently possible from *E. coli* hosts, and it is possible that secreted IL-2 from yeast may contain non-native glycosylation resulting from post-translational modification.

It is also, of course, possible to express genes encoding polypeptides in non-human eucaryotic host cell cultures derived from multicellular organisms. See, for example, Tissue Cultures, Academic Press, Cruz and Patterson, editors (1973). Useful host cell lines include VERO, HeLa cells, Chinese hamster ovary (CHO) cells, as well as fungal systems such as *Aspergillus*. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al., Nature (1978) 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. General aspects of mammalian cell host system transformations have been described by Axel; U.S. Patent No. 4,399,216 issued August 16, 1983. It now appears, also, that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes. Plant cells are also now available as hosts, and control sequences compatible with plant cells such as the nopaline synthase promoter and polyadenylation signal sequences - (Depicker, A., et al., J. Mol. Appl. Gen. (1982) 1:561) are available.

## C.2 Transformations

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S. N., Proc. Natl. Acad. Sci. (USA) (1972) 69 :2110, or the RbCl<sub>2</sub> method described in Maniatis, et al., Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press, p. 254 is useful for prokaryotes or other cells which contain substantial cell wall barriers. Infection with *Agrobacterium tumefaciens* (Shaw, C. H., et al., Gene (1983) 23:315) is used for certain plant cells. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546 is preferred. Transformations into yeast are carried out according to

the method of Van Solingen, P., et al., J. Bact. - (1977) 130:946, Hsiao, C. L., et al., Proc. Natl. Acad. Sci. (USA) (1979) 76:3829, and Klebe, R. J. et al., Gene (1983) 25 :333.

## C.3 Probing cDNA or Genomic Libraries

cDNA or genomic libraries are screened using the colony hybridization procedure. Each microtiter plate is replicated onto duplicate nitrocellulose filter papers (S & S type BA-85) and colonies are allowed to grow at 37°C for 14-16 hours on L agar containing 50 µg/ml Amp. The colonies are lysed and DNA is fixed to the filter by sequential treatment for five minutes with 500 mM NaOH, 1.5 M NaCl. The filters are washed twice for five minutes each time with 5 x standard saline citrate (SSC). Filters are air dried and baked at 80°C for two hours. The duplicate filters are prehybridized at the desired temperature for a desired time period, with 10 ml per filter of DNA hybridization buffer (5 x SSC, pH 7.0 5 x Denhardt's solution (polyvinylpyrrolidone, plus Ficoll and bovine serum albumin; 1 x = 0.02% of each), 50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 20 µg/ml poly U, and 50 µg/ml denatured salmon sperm DNA).

The samples are hybridized with kinased probe under conditions which depend on the stringency desired. Typical moderately stringent conditions employ a temperature of 42°C for 24-36 hours with 1-5 ml/filter of DNA hybridization buffer containing probe. For higher stringencies higher temperatures and shorter times are employed; for lower stringencies, the reverse. The filters are washed four times for 30 minutes each time at 37°C with 2 x SSC, 0.2% SDS and 50 mM sodium phosphate buffer at pH 7, then are washed twice with 2 x SSC and 0.2% SDS, air dried, and are autoradiographed at -70°C for two to three days.

## C.4 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., N w

England Biolabs Product Catalog. In general, about 1  $\mu$ g of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20  $\mu$ l of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol followed by running over a Sephadex G-50 spin column. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction-cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM MgCl<sub>2</sub>, 6 mM DTT and 5-10  $\mu$ M dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated followed by running over a Sephadex G-50 spin column. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides are prepared by the triester method of Matteucci, et al., (J. Am. Chem. Soc. (1981) 103:3185) or using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 0.1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles  $\gamma$ -32P-ATP (2.9 mCi/mmmole), 0.1 mM spermidine, 0.1 mM EDTA.

Ligations are performed in 15-30  $\mu$ l volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 33  $\mu$ g/ml BSA, 10 mM-50 mM NaCl, and either 40  $\mu$ M ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C

(for "blunt end" ligation) Intermolecular "sticky end" ligations are usually performed at 33-100  $\mu$ g/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations - (usually employing a 10-30 fold molar excess of linkers) are performed at 1  $\mu$ M total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na<sup>+</sup> and Mg<sup>++</sup> using about 1 unit of BAP per  $\mu$ g of vector at 60°C for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated and desalted by application to a Sephadex G-50 spin column. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

#### C.5 Verification of Construction

In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming E. coli strain MM294 obtained from E. coli Genetic Stock Center, CGSC #6135, or other suitable host, with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al., Proc. Natl. Acad. Sci. (USA) (1969) 62:1159, optionally following chloramphenicol amplification (Clewell, D. B., J. Bacteriol. (1972) 110:667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., et al., Proc. Natl. Acad. Sci. (USA) (1977) 74:5463 as further described by Messing, et al., Nucleic Acids Res. (1981) 9:309, or by the method of Maxam, et al., Methods in Enzymology (1980) 65:499.

#### C.6 Hosts Exemplified

Host strains used in cloning and expression herein are as follows:

For cloning and sequencing, and for expression of construction under control of most bacterial promoters, E. coli strain MM294 (supra), Talmadge, K., et al., Gene (1980) 12:235; Meselson, M., et al., Nature (1968) 217:1110, was used as the host.

For M13 phage recombinants, *E. coli* strains susceptible to phage infection, such as *E. coli* K12, DG98, are employed. The DG98 strain has been deposited with ATCC on July 13, 1984 and has accession number 39,768. For yeast transformations, *S. cerevisiae* strains such as C468 - (Innis, M. A. et al., *Science* (1985) 228:21-26 and its cir<sup>+</sup> derivative were used. C468 cir<sup>+</sup> was deposited with ATCC on December 13, 1985 and has ATCC accession no. 20,787.

In the examples which follow, all temperatures are provided in degrees Celsius and all parts and percentages are by weight, unless otherwise indicated. These examples are meant only to typify the procedure, and are not to be construed as limiting. Therefore, other reference forms of IL-2 may be used, and other expression systems may be used to provide the protein. For example, a yeast construct can be designed to result in an intracellular yeast product lacking the leader sequence described herein. As stated above, desired coding sequences may be expressed in a variety of procaryotic and eucaryotic host systems using known control sequences.

#### EXAMPLES

##### D. Expression of Des-ala, Ala<sub>104</sub> Ser<sub>125</sub> IL-2 in *E. coli*

The following specific example is used to illustrate the application of the process of the invention to a particular active form of IL-2 as reference protein. In this example, the resulting coding sequence having the appropriate substitution to gen-

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	150 mM
KH <sub>2</sub> PO <sub>4</sub>	21.6 mM
Na <sub>3</sub> Citrate	1.5 mM
ZnSO <sub>4</sub> · 7 H <sub>2</sub> O	30 μM
MnSO <sub>4</sub> · H <sub>2</sub> O	30 μM
CuSO <sub>4</sub> · 5 H <sub>2</sub> O	1 μM

The pH was adjusted to 6.50 with 2.5 N NaOH and the mixture autoclaved.

erate an alanine codon at position 104 in place of that for methionine is ligated into an expression vector utilizing the trp promoter system and the resulting vector is used to produce the desired protein in *E. coli*. (Des-ala, designates that the alanine at the 1 position of IL-2 has been deleted).

##### D.1. Identification of Methionine Residues in IL-2 Preferentially Susceptible to Chloramine T

The IL-2 mutein, des-ala,ser<sub>125</sub> IL-2, was used as reference protein. This protein was prepared and purified to apparent homogeneity using preparative scale techniques, but was found to be heterogeneous when more sensitive analytical methods were applied. Analysis of the various species permitted identification of methionine 104 in IL-2 as the susceptible residue whose conversion to the sulfoxide accounts for much of the heterogeneity in the final product.

##### D.1.a. Preparation of Reference IL-2 in *E. coli*

Des-ala,ser<sub>125</sub> IL-2 is a biologically active mutein which contains the four methionine residues of the native sequence. It was prepared from *E. coli* K12 strain MM294 transformed with pLW45, an expression vector containing the coding sequence for this protein under control of the trp promoter. This transformed strain was deposited in the American Type Culture Collection on March 6, 1984, and has ATCC accession no. 39,626.

*E. coli* transformed with pLW45 were grown as follows:

The culture medium contained

Under sterile conditions (post autoclave), the following were added to give final concentrations as shown:

MgSO <sub>4</sub>	3 mM
FeSO <sub>4</sub>	100 μM
L-tryptophan	14 mg/l
Thiamine-HCl	20 mg/l
Glucose	5 g/l
Tetracycline	5 mg/l
Ethanol	2%
Casamino acids	2%

Dow Corning Antifoam, polypropylene glycol-20% solution, glucose-50% solution, and KOH-5 N, were added on demand, and the culture was maintained in a fermenter.

The pH of the fermenter was maintained at 6.8 with 5 N KOH. Residual glucose was maintained between 5-10 g/l, dissolved oxygen at 40%, and temperature at 37±1°C. The casamino acids (20% stock solution) to a concentration of 2% were added when the OD<sub>600</sub> was about 10. Harvest was made three hours after the OD reached about 20.

#### D.I.b. Purification of the Reference Protein

The cells were lysed, and the IL-2 mutein was purified as follows:

Briefly, the harvested cells were concentrated, for example, by hollow fiber filtration, and about 20-40 g of material was resuspended in 200 ml of 50 mM Tris/1 mM EDTA, pH 8.1-8.5, and centrifuged at 3000-4000 x g for 10 minutes. The pellet was resuspended in 200 ml of Tris/EDTA buffer at 4°C and sonicated. The cell debris from the sonicate, which contains the desired protein, was recovered by centrifugation, and the pellet resuspended in 60 ml Tris/EDTA buffer at room temperature. An equal volume of 8 M urea in the same buffer was added over five minutes, while rapid stirring was maintained. After 15-30 minutes of continued slow stirring, the debris, which again contains the desired protein, was recovered by centrifugation at 12,000 x g for 15 minutes. The urea-extracted pellet was resuspended in 9 ml of 50 mM sodium phosphate, pH 6.8, 1 mM EDTA, 10 mM DTT at 20°C; 1 ml 20% SDS was added to 2% final concentration, and the suspension was mixed vigorously for five minutes. The supernatant, which now contains the desired protein, was recovered after centrifugation at 12,000 x g for 10 minutes at room temperature. The supernatant was heated to 40°C for 15 minutes to assure complete reduction of the sulfhydryl groups.

The reduced SDS extract of the urea pellet was extracted with an equal volume of 2-butanol containing 1 mM DTT at room temperature. The organic phase, adjusted to pH 8.0, was added slowly to 0.1% SDS in mM sodium phosphate, 2 mM DTT, pH 6, and stirred for 20 minutes to give a precipitate, which was recovered, suspended in 5% SDS in phosphate buffered saline, and reduced by heating as above. The resulting solution (adjusted to pH 5.5) was purified by gel filtration using a Sephacryl-200 column.

The solution was loaded onto a 2.6 cm x 95 cm S-200 column run in 50 mM sodium acetate, pH 5.5, 1 mM EDTA, 2 mM DTT, 1% SDS. The fractions containing the highest concentration of IL-2 activity and the lowest contaminant concentration were pooled, concentrated, reduced, and oxidized using 8-50 μM cupric chloride, wherein the solubilized form of IL-2 is treated with the CuCl<sub>2</sub> in the presence of air at a pH of between about 6 and 8. (The oxidation links the cysteine residues at positions 58 and 105 to the desired cystine linkage). The oxidized protein was further purified by preparative RP-HPLC, and the IL-2 peak was pooled and lyophilized following addition of mannitol to 5%. The IL-2 protein was resuspended to the original volume in 50 mM sodium phosphate buffer (pH 6.8) containing 0.1% SDS and assayed for bioactivity (using the standard HT-2 cell proliferation assay), for protein content (Lowry) and purity (by RP-HPLC and by non-reducing SDS-PAGE). The specific bioactivity was equivalent to that of native Jurkat IL-2 (4 x 10<sup>6</sup> units/mg) and purity was over 95%.

### D.1.c. Detection and Analysis of Sulfoxide-Containing Protein

When the oxidized des-ala,ser<sub>125</sub> IL-2 from 1D.1.b was subjected to analytical RP-HPLC the results shown in Figure 3a were obtained. The major IL-2-containing peak (peak B) is preceded by a smaller peak (peak A). The peak A material was shown also to be IL-2, except that it contains a methionine sulfoxide residue at position 104.

The correlation of peak A with an oxidized methionine in IL-2 was confirmed by oxidizing the sample with chloramine T under the standard conditions described above, and subjecting the oxidized sample to analytical RP-HPLC. Figure 3a shows the RP-HPLC before and Figure 3b after chloramine T oxidation. Figure 3b shows that essentially all of peak B protein of Figure 3a appears in the peak A position following chloramine T treatment. This is consistent with the foregoing interpretation, based on the known specificity of chloramine T for methionine (Schechter, Y., et al., - (supra)). Similar results were obtained in an analogous experiment, which substituted 30 mM hydrogen peroxide for chloramine T as the oxidizing agent. (See Figures 4a and 4b).

It is known that the rate of oxidation of a particular methionine depends on its position in the three-dimensional structure, and thus the methionine residues of a particular sequence will vary in susceptibility. The location of the susceptible methionine in IL-2 was obtained in two ways. In one approach, both peaks A and B from a lot which contained a particularly large amount of peak A were separated by purification on RP-HPLC, exposed to cyanogen bromide (which cleaves protein sequences at methionine, but not at methionine sulfoxide residues), and then examined by RP-HPLC protein mapping and amino acid analysis or by N-terminal sequence analysis of the entire digest. Approximately 400 µg of material from each RP-HPLC peak was dissolved in 1 ml of 70% formic acid containing 1 mg cyanogen bromide. After incubation for 16 hours in the dark, samples were diluted 10 fold with water, lyophilized, and redissolved in 1 ml 0.15 M Tris-HCl, pH 8.8, containing 1% SDS. Aliquots of 50 µl were examined by HPLC before and after reduction with dithioerythritol (DTE), which reduction was accomplished by adding 1 mg DTE and incubating for one hour at 85°C. Analysis of the results obtained showed that all methionines in peak B IL-2 are essentially unmodified, but that in peak A, only the methionine residue at position 104 of each molecule has been oxidized to the sulfoxide.

This was confirmed by analyzing trypsin digests of peak A and peak B proteins which were reduced in dithiothreitol (DTT) carboxymethylated using iodoacetate, and subjected to RP-HPLC. Results of this analysis also confirmed that peak A protein was identical with peak B protein, except for oxidation of the methionine at position 104.

### D.2 Preparation of the Coding Sequence for Des-ala, Ala<sub>104</sub> Ser<sub>125</sub> IL-2 in *E. coli*

An ala<sub>104</sub> mutein of the des-ala,ser<sub>125</sub> IL-2 of 1D.1 was prepared by site-specific mutagenesis using an M13 cloned reference protein sequence, prepared substantially as described in U.S. Patent No. 4,518,584 issued May 21, 1985.

Briefly, the IL-2 gene from the plasmid pLW1, which was deposited at the American Type Culture Collection on August 4, 1983, and assigned ATCC no. 39,405, was cloned into M13mp9 to give M13-IL-2. M13-IL-2 was used to serve as a template for oligonucleotide-directed mutagenesis to convert the cysteine at position 125 to a serine residue. To this end, 40 pmoles of the oligonucleotide 5'-GATGATGCTCTGAGAAAAGGTAATC-3' was kinased under standard conditions for use as primer and probe. Ten pmoles of the kinased primer was hybridized to 2.6 µg of single-stranded (ss) M13-IL-2 DNA in 15 µl of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM MgCl<sub>2</sub>, and 20 mM β-mercaptoethanol by heating at 67°C for five minutes and 42°C for 25 minutes. The annealed mixture was chilled on ice and adjusted to a final volume of 25 µl in a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM MgCl<sub>2</sub>, 83 mM NaCl, 17 mM β-mercaptoethanol, 5 units of DNA polymerase 1, Klenow fragment, 0.5 mM ATP, and 2 units of T<sub>4</sub> DNA ligase, incubated at 37°C for five hours. The reactions were terminated by heating to 80°C and the reaction mixtures were used to transform competent JM103 cells, which were plated onto agar plates, and incubated overnight to obtain phage plaques. The plaques were probed using kinased primer using standard prehybridization and hybridization conditions at high stringency (42°C for eight hours). A plaque which hybridized to primer was picked. This plaque was designated M13-LW46, and contains the coding sequence for des-ala,ser<sub>125</sub> IL-2.

M13-LW46 was subjected to site-specific mutagenesis in a precisely analogous process, but using the primer 5'-CAGCATACTCACACGCGAATGTTGTTTC-3'. This primer was complementary to a sequence wherein nucleotides 307 and 308 are GC, instead of AT, as

in the reference sequence. The oligonucleotide was kinased, and used as primer and probe in site-specific mutagenesis and recovery of mutagenized phage as above.

One of the mutagenized M13-LW46 plaques which hybridized with probe was designated SDL23, picked, cultured, and used to prepare expression vector pSY3001.

### D.3 Construction of pSY3001

RF-DNA from SDL23 was digested with HindIII and PstI and the insert fragments were purified from a 1% agarose gel. Similarly, pTRP3, a pBR322 derivative containing the trp promoter, - (1D.7) was digested with HindIII and EcoRI; the small fragment containing trp promoter was purified on an agarose gel. The pBR322 vector was digested with EcoRI and PstI and the large fragment purified on an agarose gel. The purified vector fragment and trp promoter fragment were ligated with the IL-2 mutein encoding fragment, and the ligation mixture was transformed into competent *E. coli* K12 strain MM294, generating a Tet<sup>R</sup> phenotype. The plasmid DNA was isolated and correct construction of pSY3001 confirmed by restriction analysis and dideoxy sequencing.

### D.4 Production and Purification of Des-ala-Ala<sub>104</sub>Ser<sub>125</sub> IL-2

*E. coli* transformed with pSY3001 were then grown as described in 1D.1.a above, and the desired oxidation-resistant mutein was purified and oxidized as described in 1D.1.b, with the following exceptions: The reduced supernatant from the 2% SDS-extracted urea pellet was loaded onto a 2.6 cm x 95 cm G-100 column run in 50 mM sodium phosphate, pH 6.8, 1 mM EDTA, 1 mM DTT, 0.1% SDS. The fractions containing IL-2 activity and the lowest contaminant concentration were pooled and concentrated by ultrafiltration using an Amicon YM-5 membrane. To ensure that all molecules were reduced, DTT was added to 10 mM, and the sample was heated to 60°C for 10 minutes. The sample was immediately desalted using a 0.9 cm x 20 cm G-25 column with 50 mM sodium phosphate - (pH 7.0), 0.1% SDS. The resulting purified oxidation-resistant mutein was immediately oxidized to obtain the cystine linkage as described above and assayed for biological activity and purity in a manner similar to that used for the reference protein, with similar results.

The standard HT-2 cell bioassay (supra) was used to assess the biological activity of the purified material from the pSY3001-transformed cells. The specific activity of the oxidation-resistant mutein was approximately 4 x 10<sup>6</sup> units/mg, the same as that for the clinical grade des-ala,ser<sub>125</sub> IL-2, of native IL-2 which was purified from peripheral blood lymphocytes or the Jurkat cell line. The oxidation-resistant mutein displayed the same biological activity as the reference IL-2 protein when stored in solution at 4°C for two months. The mutein also showed the same specific activity as the reference protein in NK activation assays.

The course of purification of the oxidation-resistant mutein is shown in Figure 4, and the purified oxidation-resistant mutein product is compared with the purified reference IL-2 protein obtained from pLW45 transformants.

### D.5 Homogeneity and Activity of the IL-2 Oxidation-Resistant Mutein

The purified oxidation-resistant mutein purified from pSY3001 transformant as in 1D.4 was subjected to analytical RP-HPLC with the results shown in Figure 2c. A single symmetrical peak was obtained showing that the peak A material which was obtained in similarly purified reference IL-2 has been eliminated. The RP-HPLC retention time of the mutein differs slightly from that of the reference protein, as has been observed for other IL-2 point mutations (Kunitani, et al., Fifth Int'l Symposium on HPLC of Proteins, Peptides and Polynucleotides, Toronto, Canada, November 4-6 1985). Figure 2d shows the results of chloramine T treatment of this preparation conducted exactly as set forth above for the reference IL-2. As expected, no change in the RP-HPLC pattern was obtained for des-ala,ala<sub>104</sub>ser<sub>125</sub> IL-2, indicating that this mutein lacks the methionine residue preferentially susceptible to oxidation by chloramine T.

Figures 3c and 3d show the corresponding results obtained when oxidation was carried out using hydrogen peroxide.

### D.7 Other Oxidation-Resistant IL-2 Muteins in *E. coli*

The DNA sequence for native IL-2, des-ala, IL-2, and ser<sub>125</sub> IL-2, as well as the above-illustrated des-ala,ser<sub>125</sub> IL-2, can be genetically modified using analogous methods, but substituting as starting materials for M13-LW46, the following: to prepare ala<sub>104</sub> IL-2, M13-LW32; to prepare des-ala,ala<sub>104</sub> IL-2, M13-IL-2; to prepare ala<sub>104</sub>ser<sub>125</sub> IL-2, an M13 vector prepared using pLW55, a plasmid containing the

desired coding sequence, which was deposited at the American Type Culture Collection on November 18, 1983, and bears ATCC accession no. 39,516. In addition, the DNA sequence for ala<sub>125</sub> IL-2 can be genetically modified using analogous methods. Ala<sub>125</sub> IL-2 has been prepared and purified and shown to be fully active in biological assays.

These starting materials are described in detail in U.S. Patent No. 4,518,584 (supra).

#### D.8 Construction of pTRP3

To construct the host vector containing the trp control sequences behind a HindIII site, the trp promoter/operator/ribosome binding site sequence, lacking the attenuator region, was derived from pVH153, obtained from C. Yanofsky, Stanford University. Trp sequences are available in a variety of plasmids well known in the art. pVH153 was treated with HhaI (which cuts leaving an exposed 3' sticky end just 5' of the trp promoter) blunt-ended with Klenow, and partially digested with TaqI. The 99 bp fragment corresponding to restriction at the TaqI site, 6 nucleotides preceding the ATG start codon of trp leader, was isolated, and then ligated to EcoRI (repair)/ClaI digested, pBR322 to provide pTRP3. pTRP3 was deposited December 18, 1984 and has ATCC accession no. 39,946.

#### E. Expression of Ala<sub>125</sub> IL-2 in Yeast

The following specific example is used to illustrate additional applications of the process of the invention for the production of alternative active forms of IL-2 in a host other than *E. coli*. In this example, coding sequences having appropriate substitutions to generate any one of several muteins are ligated into a yeast expression vector utilizing the yeast alpha factor promoter, leader, and terminator sequences, and used to produce the desired proteins in yeast. While the expression vectors for IL-2 and the reference protein were actually prepared, the isolation, purification and characterization of the muteins have not been completed, and the procedure below represents expected procedure where the present tense is used.

##### E.1 Preparation of Reference IL-2 in Yeast

Mature wild-type recombinant IL-2 containing the same amino acid sequence present in the mature IL-2 isolated from human cells was prepared from a cir<sup>o</sup> derivative of *Saccharomyces cerevisiae* strain C468 (Innis, M.A. et al., *Science* - (1985) 228:21-26) transformed with pPM42, an expression vector containing the mature native IL-2

coding sequence fused in frame at its 5' end with the yeast alpha factor signal peptide sequence under the control of the yeast alpha factor promoter. The pPM42 transformed strain was deposited in the American Type Culture Collection on December 13, 1985 and has ATCC Accession No. 53355.

A seed culture of *S. cerevisiae* transformed with pPM42 was started by thawing a frozen yeast culture (with 10% glycerol) and diluting it 1 to 50 into a selective seed medium containing:

0.01 M succinic acid

5.0 mM H<sub>3</sub>PO<sub>4</sub>

3.0 mM H<sub>2</sub>SO<sub>4</sub>

5.0 mM KCl

1.0 mM NaCl

1.0 mM MgCl<sub>2</sub> • 6H<sub>2</sub>O

0.01 mM MnSO<sub>4</sub> • H<sub>2</sub>O

1.0 μM CuSO<sub>4</sub> • 5H<sub>2</sub>O

5.0 μM ZnSO<sub>4</sub> • 7H<sub>2</sub>O

5.0 μM CoCl<sub>2</sub> • 6H<sub>2</sub>O

5.0 μM Na<sub>2</sub>MoO<sub>4</sub> • 2H<sub>2</sub>O

0.05 mM H<sub>3</sub>BO<sub>3</sub>

0.1 mM CaCl<sub>2</sub> • 2H<sub>2</sub>O

0.2 g/l histidine

The pH was adjusted to 4.25 with NH<sub>4</sub>OH. After autoclaving, the following sterile additions were made to give final concentrations as shown:

10% glucose

0.5 μg/l pyridoxine HCl

1.0 μg/l thiamine HCl

0.01 μg/l D-biotin

1.0 μg/l calcium pantothenate

0.04 g/l myo-inositol (meso-inositol)

0.04 mM FeSO<sub>4</sub>

This culture was grown aerobically at 30°C for 2-3 days until the cell density measured by A<sub>680nm</sub> reached 10-20.

This culture was then used to inoculate a larger fermenter containing 10 liters of medium. This medium consisted of:

75 mM NH<sub>4</sub>Cl

5.0 mM H<sub>3</sub>PO<sub>4</sub>

3.0 mM H<sub>2</sub>SO<sub>4</sub>

5.0 mM KCl

1.0 mM NaCl

1.0 mM MgCl<sub>2</sub> • 6H<sub>2</sub>O

0.01 mM MnSO<sub>4</sub> • H<sub>2</sub>O

1.0 μM CuSO<sub>4</sub> • 5H<sub>2</sub>O

5.0 μM ZnSO<sub>4</sub> • 7H<sub>2</sub>O

5.0 μM CoCl<sub>2</sub> • 6H<sub>2</sub>O

5.0 μM Na<sub>2</sub>MoO<sub>4</sub> • 2H<sub>2</sub>O

0.05 mM H<sub>3</sub>BO<sub>3</sub>

0.1 mM CaCl<sub>2</sub> • 2H<sub>2</sub>O

0.5 g/l histidine

The pH was adjusted to 5 with 2.5 N NaOH and the mixture was autoclaved. Under sterile conditions - (post autoclave), the following additions were made to give final concentrations as shown:

10-15% glucose

0.5 μg/l pyridoxine HCl

1.0 μg/l thiamine HCl

0.01 μg/l D-biotin

1.0 μg/l calcium pantothenate

0.04 g/l myo-inositol (meso-inositol)

0.04 mM FeSO<sub>4</sub>

Dow Coming Silicon Emulsion B was added to the fermentor on demand to control foaming. The pH of the fermenter was maintained at 4.5 by addition of 4N NaOH, and dissolved oxygen was held at 40% of air saturation by air sparging. The fermenter was agitated at 500 rpm and maintained at 30°C for 2-3 days until the culture density reached an A<sub>680nm</sub> of about 20. The cultures were harvested by cell separation and further processing of the culture supernatant.

## E.2. Suggested Purification of the Reference Protein from Yeast

Cells are separated from the culture supernatant by centrifugation at 4000 x g for 15 min. or by cross-flow filtration.

The previously described clarified yeast culture supernatant is then concentrated using a hollow fiber cartridge with a permeability to molecules of molecular mass of 10,000 daltons or less only (e.g., PM10). After concentrating the IL-2-containing supernatant approximately 100-200 fold, SDS is added to 2% and the solution is heated to 37°C for 20 min. The unpurified IL-2 solution is then applied to a Sephacryl-200 column (2.6 x 95 cm) which has been previously equilibrated in 50 mM sodium phosphate pH 7.0 containing 0.2 M NaCl and 0.1% SDS. The elution position of the IL-2 protein is visualized by SDS-PAGE analysis.

The IL-2 peak fractions are pooled and concentrated on an Amicon concentrator using a PM10 membrane. Acetonitrile is then added to 10% and the pH is reduced to about 3 by the addition of TFA to 0.5% (vol/vol). The IL-2 solution is loaded onto a Vydac C<sub>4</sub> column (10 mm x 25 cm) and eluted with an acetonitrile gradient of 30-60% in 45 min at a flow rate of 2 ml/min. The IL-2 peak, which elutes later than most of the other contaminating proteins, is diluted 2-fold with water and rechromatographed on a second Vydac C<sub>4</sub> column at a flow rate of 1 ml/min but using otherwise similar conditions to those previously described. The purified IL-2 is then formulated as described above, either in the presence or absence of detergent.

## E.3. Detection and Analysis of Methionine Sulfoxide-Containing Protein from Yeast

When the supernatant from a culture of *S. cerevisiae* transformed with pPM42 and grown as described in 1E.1 was analyzed by RP-HPLC, two peaks of biologically active IL-2 proteins were resolved. The early eluting peak, peak A, constituted approximately 15% of the total IL-2 bioactivity recovered. The remainder of IL-2 eluted somewhat



later, in peak B. Both IL-2 peaks had approximately equivalent biological specific activity in the HT-2 cell proliferation assay described in 1B.1. The RP-HPLC profile was similar to that of IL-2 produced in *E. coli* in which the early eluting peak, peak A, was shown to contain a methionine sulfoxide residue at position 104 instead of a methionine found at the same position in the peak B material.

When an identical purification was carried out on a different aliquot of the same yeast culture supernatant described above which had been previously stored at 4°C for two weeks, RP-HPLC analysis revealed that about 60% of the IL-2 preparation eluted at the peak A position. This result suggested that an oxidant was present in the yeast culture supernatant which, upon storage, was continuing to oxidize the methionine at position 104 in the IL-2 protein.

SDS-PAGE analysis of RP-HPLC peak A and peak B material indicated identical molecular weights for the IL-2 from both peaks. This result made the possibility that proteolysis or differences in glycosylation were the cause of the shift in RP-HPLC retention time much less likely. In addition, experiments performed using specific chemical oxidants such as chloramine T indicate that peak A arises from peak B by the oxidation of a specific methionine residue to methionine sulfoxide. The notion that peak A results from the oxidation of the methionine residue at position 104 in peak B IL-2 from yeast are confirmed by the absence of peak A material in muteins containing alanine at position 104 in place of methionine.

#### E.4. The Coding Sequence for Ala<sub>104</sub>-Containing Muteins IL-2 in Yeast

An ala<sub>104</sub> mutein of the wild-type IL-2 of 1E.1 was prepared by site-specific mutagenesis using an M13 cloned reference protein sequence, prepared substantially as described in U.S. Patent No. 4,518,584, issued May 21, 1985, and the disclosure of which is incorporated herein by reference. The IL-2 ala<sub>104</sub>ala<sub>125</sub> mutein may be obtained by the same procedure and the work is underway.

Briefly, the IL-2 gene from the plasmid pLW32, which was deposited at the American Type Culture Collection on December 13, 1985 and assigned ATCC No. 53,354, was excised as a *Hind*III-*Stu*I fragment (Wang, A. et al. (1984) *Science* 224:1431-1433; the *Stu*I site is located some 132 bp upstream from the *Bam*II site). A *Hind*III linker was added to the blunt *Stu*I end of the fragment. This provided a *Hind*III fragment containing the IL-2 gene for insertion into an M13mp7 vector which had been modified as follows.

An approximately 1.5 kb *Eco*RI fragment containing the yeast mating factor alpha 1 gene - (Singh, A. et al. (1983) *Nucleic Acids Res.* 11:4049-4063) from which the *Hind*III to *Sal*I fragment - (nucleotides 268 to 533) had been deleted to remove the coding sequence for the four copies of the mature alpha-factor, was inserted between the *Eco*RI sites of M13mp7. Ligation of the *Hind*III end located 3' of the alpha-factor leader sequence - (nucleotide 267) to the repaired *Sal*I end following the fourth copy of the mature alpha-factor coding sequence (nucleotide 534) resulted in the creation of a unique *Hind*III site in the modified M13mp7 vector (and deletion of the mature alpha-factor coding sequences as just mentioned). The thus modified M13mp7 was designated M13mp7::MFalpha-delta and contained the yeast alpha factor promoter, leader, and terminator sequences with a unique *Hind*III site between the leader and terminator into which a gene of interest could be inserted. M13mp7::MFalpha-delta was deposited at the American Type Culture Collection on December 13, 1985 and assigned ATCC No. 40,210.

The *Hind*III fragment carrying the IL-2 gene previously described was then inserted into M13mp7::MFalpha-delta at its unique *Hind*III site. An oligonucleotide primer of the following sequence

5'CTTTGGATAAAAGAGCGCCTACTTCAAG3' was utilized to delete some 16 nucleotides in order to bring the alpha-factor leader peptide sequence and the IL-2 coding sequence into juxtaposition such that the amino acid sequence at the junction was Lys Arg Ala, which can be correctly recognized as a processing site. The resulting construct was designated M13mp7::MFalpha-delta (IL-2), deposited on December 13, 1985 at the ATCC and assigned ATCC No. 40,211.

The *Eco*RI fragment from M13mp7::MFalpha-delta (IL-2) carrying the alpha-factor promoter, leader, IL-2 gene and alpha-factor terminator was excised and the *Eco*RI ends were made blunt by repair with DNA polymerase I described in 1C.4 of the instant application. The plasmid, pJDB219, capable of replicating in both *E. coli* and yeast, has been described (Beggs, J.D. (1978) *Nature* 275:104-109) and contains two *Tth*I sites in a non-essential region of the portion of pJDB219 originally derived from *E. coli* plasmid pMB9 (see Figure 6). The vector *Tth*I fragment was then replaced by the blunt-ended *Eco*RI fragment carrying the IL-2 gene. Two orientations of the fragment within the vector are obtained by this method but no difference has been detected between them with regard to IL-2 gene expression. The production of wild type IL-2 was carried out using the construc-

tion designated pPM42, in which the IL-2 gene is in the same orientation as the tetracycline resistance - (Tet<sup>R</sup>) gene in pJDB219 (i.e., clockwise in Figure 6). This plasmid, pPM42, was deposited at the American Type Culture Collection on December 13, 1985 and assigned ATCC No. 53,355.

Ala<sub>104</sub>-containing muteins of IL-2 in yeast were obtained by site-specific mutagenesis using M13mp7::MFalpha-delta (IL-2) as template. To convert the methionine at position 104 to alanine, the oligonucleotide 5'-ACAACATTCGCTTGTGAATATG-3' was synthesized and used substantially as described in 1D.2. The cysteine at position 125 was converted to alanine using the oligonucleotide primer 5'-GATTACCTTCGCTCAAAGCATC-3'. To produce genes containing more than one modification, successive rounds of mutagenesis each using the appropriate primer were carried out.

#### E.5. Construction of Expression Vectors Containing Various Ala<sub>104</sub> IL-2 Muteins

Following mutagenesis and selection of the correctly modified coding sequence in M13mp7::MFalpha-delta (IL-2), the IL-2 mutein gene and its control sequence were removed as an EcoRI fragment as described in 1E.4 for the wild-type IL-2 gene fragment. After DNA polymerase I repair, the blunt-ended fragment containing the IL-2 mutein gene was ligated into pJDB219 between the two TthI sites, thereby replacing the vector fragment. The expression vector that resulted containing the ala<sub>104</sub> IL-2 mutein was designated pPM43 and was deposited at the American Type Culture Collection on December 13, 1985 and assigned ATCC No. 53,356.

The plasmid DNAs isolated initially from E. coli containing the above described construction and pPM42 described in 1E.4 were transformed into polyethylene glycol (PEG)-treated S. cerevisiae strain C468 cir<sup>+</sup> as detailed below to generate a leu<sup>+</sup> phenotype. Cells to be transformed were prepared substantially as described in Klebe, R.J. et al. (1983) Gene 25:333-341. Briefly, a single colony was picked into 2 ml YEPD (10 g/l yeast extract; 20 g/l peptone; 2% glucose) and grown overnight at 30°C. The overnight culture was diluted 80-fold into fresh YEPD to provide 10 ml of diluted culture per transformation. Cultures were grown at 30°C to A<sub>600 nm</sub> = .6-.9, usually about 3-3.5 hrs. Cells were pelleted by a 5 min centrifugation at 4000 rpm - (Sorvall JA-20 rotor) at room temperature. Cell pellets were each resuspended in 5 ml SBEG (1M sorbitol, 20 mM bicine pH 8.35, 3% ethylene glycol) and centrifuged again. Cell pellets were each

resuspended in 0.2 ml SBEG for 5 min at room temperature. Five to ten µg of transforming DNA was added in no more than 20 µl and the mixture was incubated 10 min at 30°C. The mixture was frozen at -70°C for at least 10 min. The mixture was thawed in a 37°C bath and 1.5 ml PEG-bicine (40% PEG-1000; 200 mM bicine pH 8.35) was added. After mixing gently, the transformations were incubated 60 min at 30°C. With gentle mixing, 3 ml NB (150 mM NaCl; 10 mM bicine pH 8.35) was added slowly, and the cells were pelleted by centrifugation in a tabletop centrifuge for 3 min at 2000 rpm. Finally, cells were resuspended in 1 ml NB and plated directly on selective medium (in this case 1.45 g/l Yeast Nitrogen Base (Difco); 0.04 M(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 2% glucose; amino acids minus leucine).

To assay the IL-2 bioactivity of yeast transformants, single yeast colonies from selective plates were picked into 3 ml of either selective or non-selective medium and incubated overnight with shaking at 30°C. An aliquot of the culture was removed and filtered through a 0.2 micron filter (Gelman acrodisc) to remove all yeast cells. Supernatants were diluted without further treatment for assay of biological activity as previously described (supra).

#### E.6. Suggested Production and Purification of Ala<sub>104</sub>-Containing IL-2 Muteins from Yeast

S. cerevisiae transformed with pPM43 is then grown as described in 1E.1 above, and the desired oxidation-resistant mutein is purified as described in 1E.2 above.

#### E.7. Predicted Biochemical and Biological Characterization of the Oxidation-Resistant Mutein from Yeast

Yeast cells containing the plasmid pPM43 are grown according to the procedure described in 1E.1 and ala<sub>104</sub>IL-2 is purified from the culture supernatant as described in 1E.2. Yeast ala<sub>104</sub>IL-2 is fully active when compared to the reference protein in biological assays such as activation of NK cells, or the IL-2 dependent HT-2 cell proliferation assay. RP-HPLC analysis of the purified oxidation-resistant mutein shows a single symmetrical peak, demonstrating that the peak A material which was obtained in similarly purified reference IL-2 has been eliminated. The RP-HPLC retention time of the mutein differs slightly from that of the reference protein, as has been observed for other IL-2 point

mutations, including des-ala<sub>1</sub>ala<sub>104</sub>ser<sub>125</sub>IL-2 in E. coli (Kunitani, et al., Fifth Int'l Symposium on HPLC of Proteins, Peptides, and Polynucleotides, Toronto, Canada, November 4-6, 1985).

Chloramine T treatment of this preparation conducted exactly as set forth above for the reference IL-2 shows no change in the RP-HPLC pattern, indicating that this mutein lacks the methionine residue preferentially susceptible to oxidation by chloramine T.

At least any one of the first five amino acids may be deleted, in any combination, or substituted by another amino acid, at the N-terminus of the oxidation-resistant IL-2 mutein with retention of biological activity.

In addition, a substitution of a conservative amino acid such as alanine or serine at position 125 of the IL-2 mutein may be made to confer additional stability on the mutein, by replacing cysteine residues which are not essential to the biological activity of the mutein. Any permutation of any of these changes alone or in combination with one or more other of these changes may be made to the oxidation-resistant muteins herein. For example, the following muteins are within the scope of this invention:

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ala<sub>104</sub>ser<sub>125</sub>IL-2, ala<sub>104</sub>IL-2, ala<sub>104</sub>ala<sub>125</sub>IL-2, glu<sub>104</sub>ser<sub>125</sub>IL-2,  
 glu<sub>104</sub>IL-2, glu<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>ala<sub>104</sub>ser<sub>125</sub>IL-2, des-  
 ala<sub>1</sub>ala<sub>104</sub>IL-2, des-ala<sub>1</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>glu<sub>104</sub>ser<sub>125</sub>IL-2,  
 des-ala<sub>1</sub>glu<sub>104</sub>IL-2, des-ala<sub>1</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-  
 pro<sub>2</sub>ala<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>ala<sub>104</sub>IL-2, des-ala<sub>1</sub>des-  
 pro<sub>2</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>glu<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-  
 pro<sub>2</sub>glu<sub>104</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-  
 thr<sub>3</sub>ala<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>ala<sub>104</sub>IL-2, des-ala<sub>1</sub>des-  
 pro<sub>2</sub>des-thr<sub>3</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>glu<sub>104</sub>ser<sub>125</sub>IL-  
 2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>glu<sub>104</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-  
 thr<sub>3</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>ala<sub>104</sub>ser<sub>125</sub>IL-  
 2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>ala<sub>104</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-  
 thr<sub>3</sub>des-ser<sub>4</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-  
 ser<sub>4</sub>glu<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>glu<sub>104</sub>IL-2, des-  
 ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-  
 thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>ala<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-  
 ser<sub>4</sub>des-ser<sub>5</sub>ala<sub>104</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 ser<sub>5</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 ser<sub>5</sub>glu<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 ser<sub>5</sub>glu<sub>104</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 ser<sub>5</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 ser<sub>6</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 ser<sub>6</sub>ala<sub>104</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 ser<sub>6</sub>ala<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 ser<sub>6</sub>glu<sub>104</sub>ser<sub>125</sub>IL-2, des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 ser<sub>6</sub>glu<sub>104</sub>IL-2, and des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 ser<sub>6</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2.

Applicants have deposited the following cul-  
 tures with the American Type Culture Collection, 45  
 Rockville, Maryland, USA (ATCC):

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<u>Strain</u>	<u>ATCC Deposit No.</u>	<u>Deposit Date</u>
pLW55	39,516	11/18/83
pTRP3	39,946	12/18/84
pSY3001 in <u>E. coli</u> K12 strain MM294	39,949	12/19/84
M13mp7::MF alpha-delta	40,210	12/13/85
M13mp7::MF alpha-delta (IL-2)	40,211	12/13/85
pLW32	53,354	12/13/85
ppM42	53,355	12/13/85
ppM43	53,356	12/13/85
<u>S. cerevisiae</u> C468 cir <sup>o</sup>	20,787	12/13/85

These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between applicants and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be

promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

#### Claims

1. An oxidation-resistant mutein having interleukin-2 (IL-2) activity and in which each methionine residue of IL-2 susceptible to chloramine T or peroxide oxidation is replaced by a conservative amino acid and additional, non-susceptible methionine residues are not so replaced.

2. A mutein according to claim 1, wherein the conservative amino acid is glycine, alanine, serine, threonine, valine, isoleucine, leucine, asparagine, glutamine, glutamate, tyrosine, or phenylalanine.

3. A mutein according to claim 1 which is

ser<sub>4</sub>des-ser<sub>5</sub>ala<sub>104</sub>IL-2,  
 ser<sub>5</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2,  
 ser<sub>5</sub>glu<sub>104</sub>ser<sub>125</sub>IL-2,  
 ser<sub>5</sub>glu<sub>104</sub>IL-2,  
 ser<sub>5</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2,  
 ser<sub>6</sub>ala<sub>104</sub>ala<sub>125</sub>IL-2,  
 ser<sub>6</sub>ala<sub>104</sub>IL-2,  
 ser<sub>6</sub>ala<sub>104</sub>ser<sub>125</sub>IL-2,  
 ser<sub>6</sub>glu<sub>104</sub>ser<sub>125</sub>IL-2,  
 ser<sub>6</sub>glu<sub>104</sub>IL-2, or  
 ser<sub>6</sub>glu<sub>104</sub>ala<sub>125</sub>IL-2.

4. A mutein according to claim 1 which is

ala<sub>104</sub>ser<sub>125</sub>IL-2, ala<sub>104</sub>IL-2,

ala<sub>104</sub>ala<sub>125</sub>IL-2, des-ala<sub>104</sub>ala<sub>125</sub>IL-2,

des-ala<sub>104</sub>ser<sub>125</sub>IL-2 or

des-ala<sub>104</sub>ala<sub>125</sub>IL-2.

5. A pharmaceutical or veterinary formulation comprising a mutein according to any one of claims 1 to 4 formulated for pharmaceutical or veterinary use, and optionally an inert, non-allergenic, compatible carrier.

6. A method for preparing an oxidation resistant mutein of a protein having interleukin-2 (IL-2) activity comprising substituting a conservative amino acid for each methionine residue susceptible to oxidation in said protein and wherein additional, non-susceptible methionine residues are not so substituted.

7. A method according to claim 6, wherein the

des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-  
 des-ala<sub>1</sub>des-pro<sub>2</sub>des-thr<sub>3</sub>des-ser<sub>4</sub>des-ser<sub>5</sub>des-

20 conservative amino acid is glycine, alanine, serine, threonine, valine, isoleucine, leucine, asparagine, glutamine, glutamate, tyrosine, or phenylalanine.

25 8. A recombinant DNA sequence which codes for an oxidation-resistant mutein according to any one of claims 1 to 4.

30 9. A recombinant expression vector comprising a DNA sequence according to claim 8 operably linked to control sequences compatible with a chosen host.

35 10. A recombinant expression vector according to claim 9 comprising a gene coding for ala<sub>104</sub>IL-2 which is designated PPM43 and is obtainable from ATCC No. 53356.

40 11. A chosen host cell transformed with an expression vector according to claim 9 or claim 10.

45 12. A method of producing a mutein according to any one of claims 1 to 4 comprising culturing cells of a chosen host transformed with a recombinant expression vector according to claim 9 or claim 10.

50

55

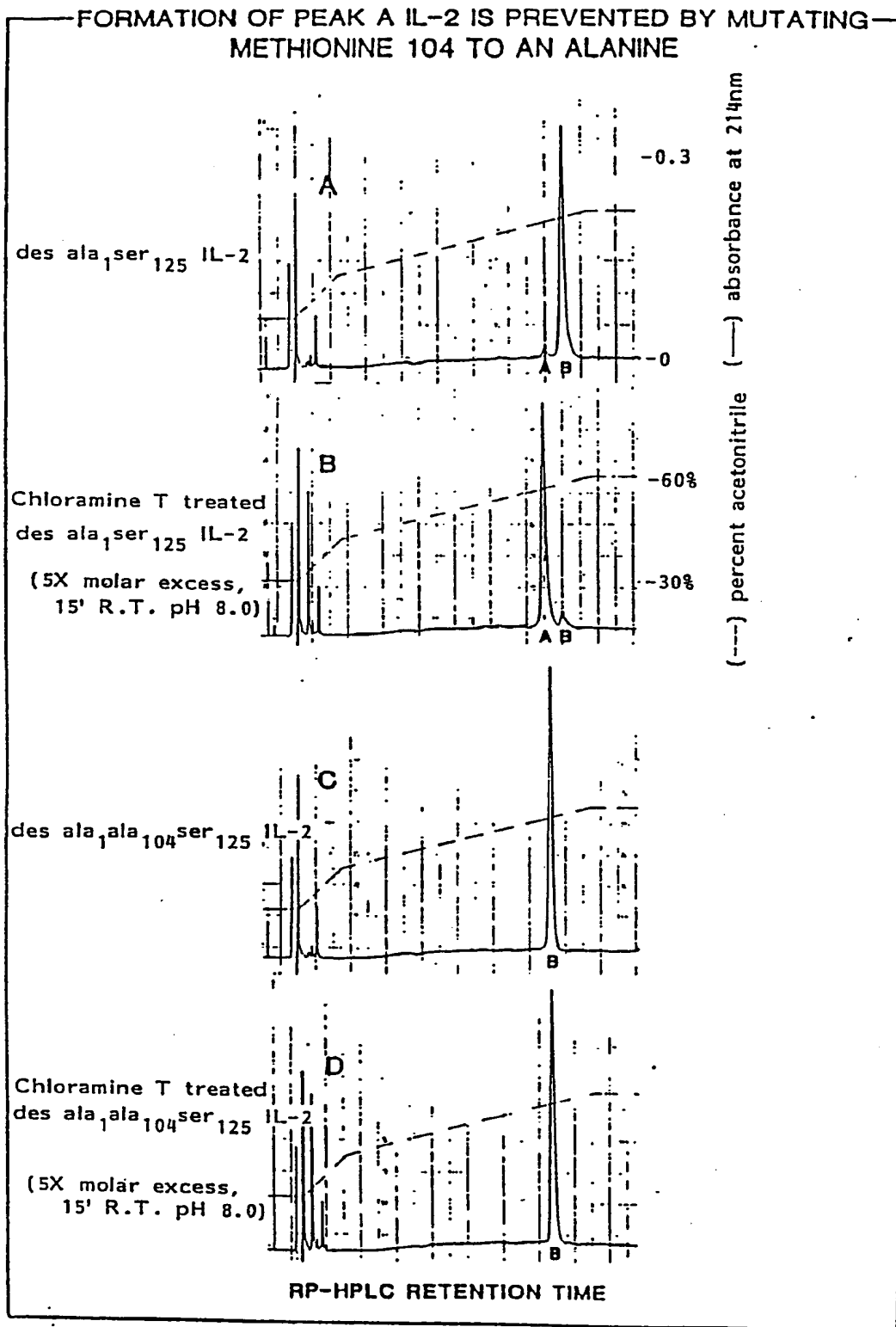
22

AlaProThrSerSer	5	SerThrLysLysThr	10	GlnLeuGlnLeuGlu	15	HisLeuLeuLeuAsp	20
LeuGlnMetIleLeu	25	AsnGlyIleAsnAsn	30	TyrLysAsnProLys	35	LeuThrArgMetLeu	40
ThrPheLysPheTyr	45	MetProLysLysAla	50	ThrGluLeuLysHis	55	LeuGlnCysLeuGlu	60
GluGluLeuLysPro	65	LeuGluGluValLeu	70	AsnLeuAlaGlnSer	75	LysAsnPheHisLeu	80
ArgProArgAspLeu	85	IleSerAsnIleAsn	90	ValIleValLeuGlu	95	LeuLysGlySerGlu	100
ThrThrPheMetCys	105	GluTyrAlaAspGlu	110	ThrAlaThrIleVal	115	GluPheLeuAsnArg	120
TrpIleThrPheCys	125	GlnSerIleIleSer	130	ThrLeuThr----	135		140

# FIG 1

MATURE NATIVE IL-2

AMINO ACID SEQUENCE





FORMATION OF PEAK A IL-2 IS PREVENTED BY  
MUTATING METHIONINE 104 TO AN ALANINE

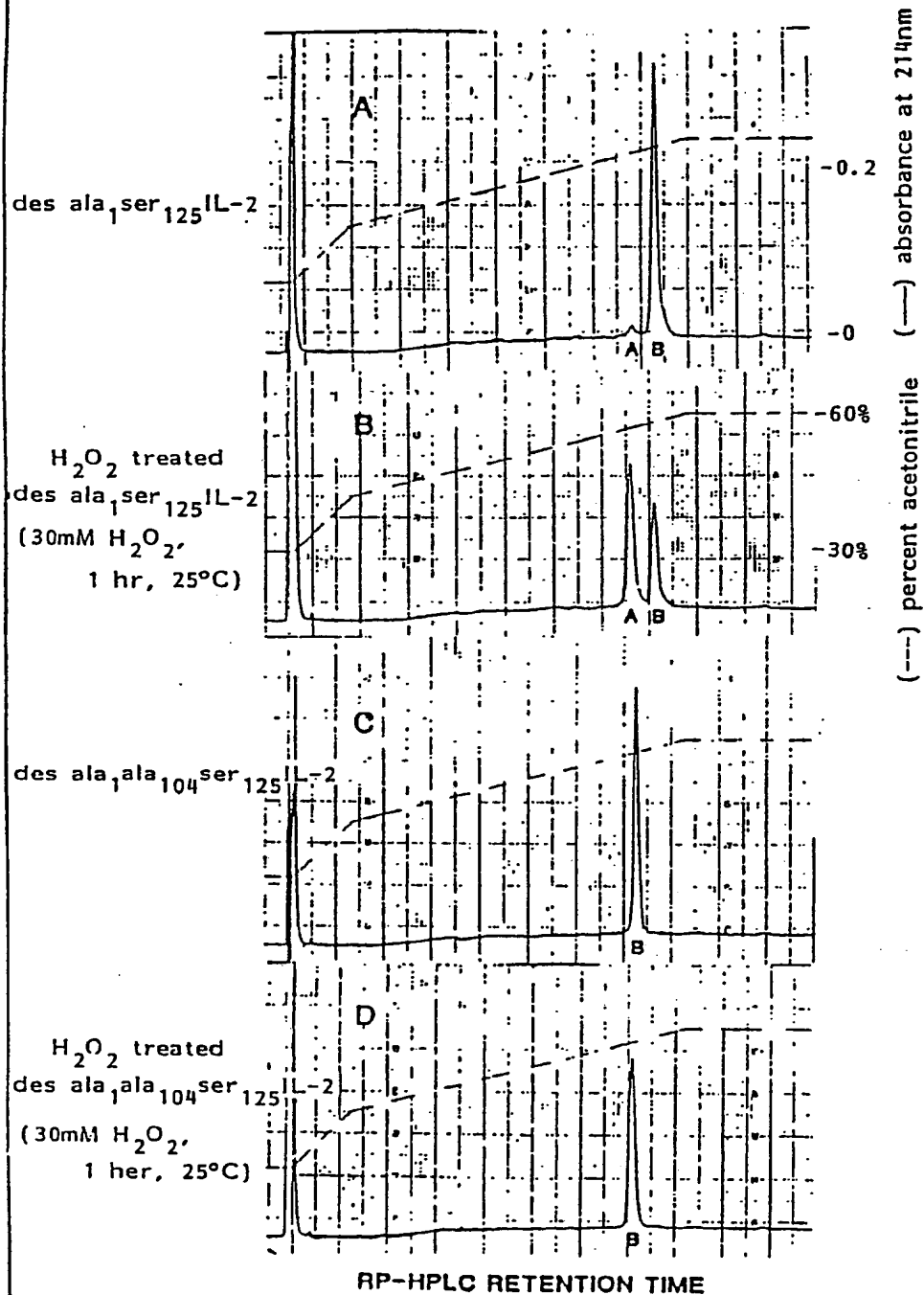
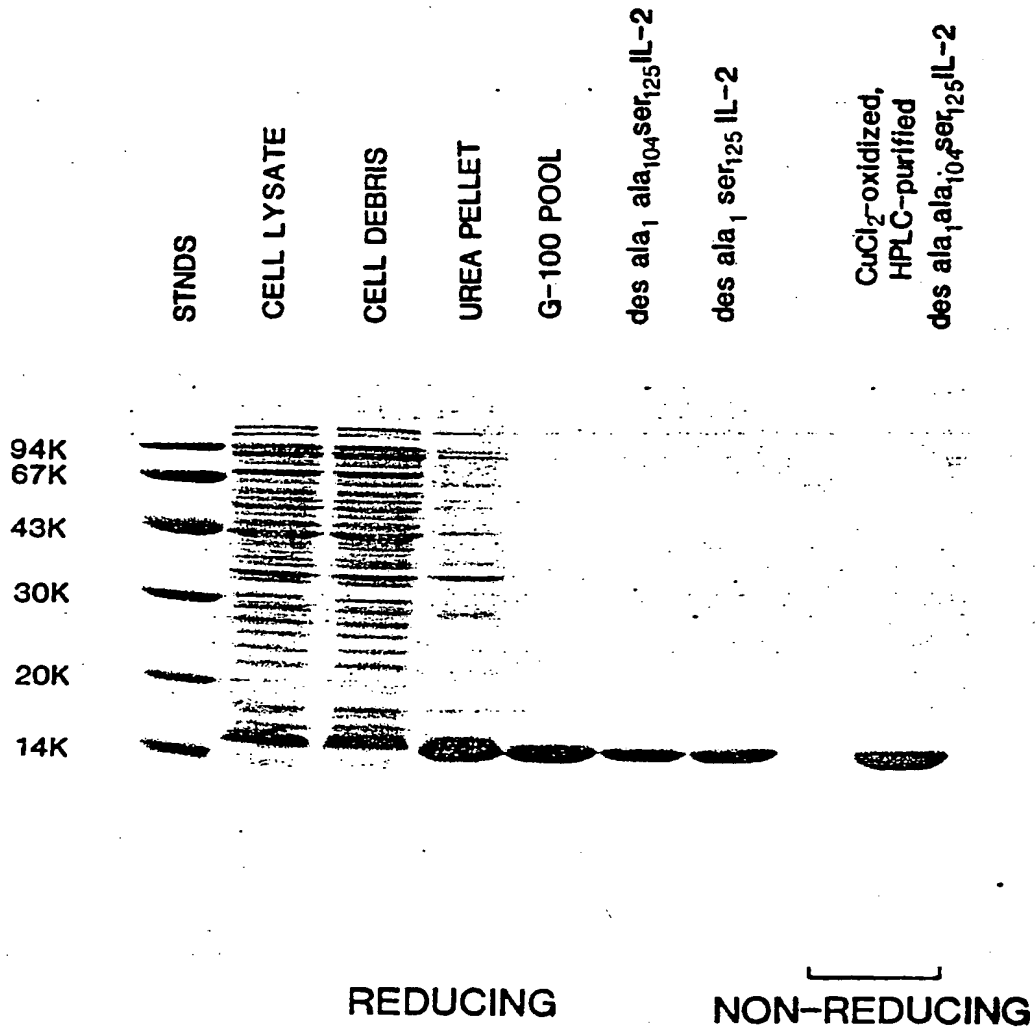


FIG 3



14% SDS-PAGE  
COOMASSIE STAINED

PURIFICATION OF A NEW IL-2 MUTANT  
(ALA 104)  
FIG 4

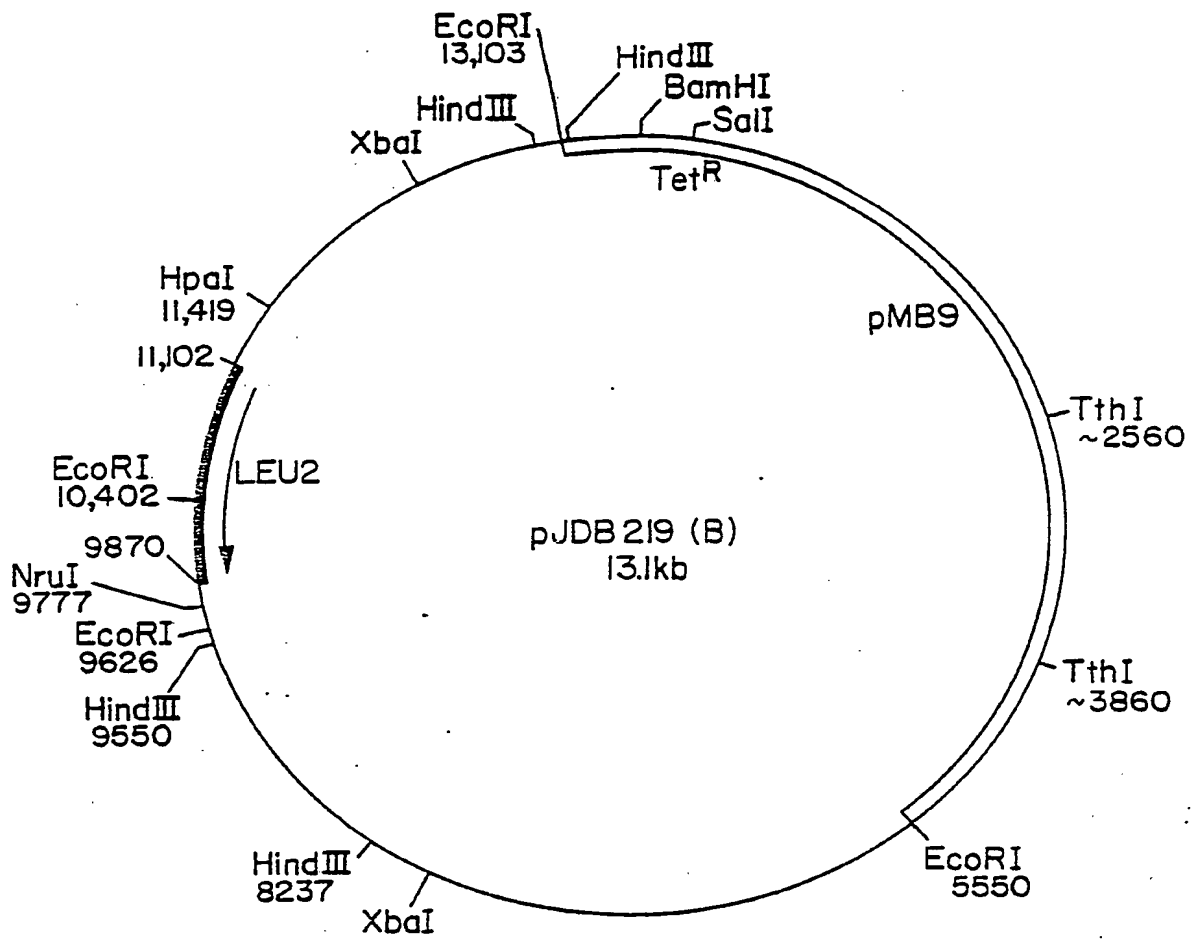


FIG 5